Journal of Fluorescence, Vol. 13, No. 5, September 2003 (©2003)

Membrane Fluidity of the Pentachlorophenol-Mineralizing *Sphingomonas* sp. UG30

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Received April 1, 2003; accepted June 18, 2003

The effect of PCP and NaPCP on the cytoplasmic membranes of the PCP-mineralizing bacterium *Sphingomonas* sp. UG30 was assessed using fluorescence polarization and total cellular fatty acid analysis. Direct exposure of resting UG30 cells to PCP up to 250 ppm and NaPCP up to 1000 ppm did not cause any changes in the polarization ratios or the fatty acid profile of the UG30 cytoplasmic membranes. Growth of UG30 cells in the presence of 25 ppm NaPCP did not affect the total cellular fatty acid profile or membrane fluidity as observed by fluorescence polarization.

KEY WORDS: Bacteria; fluorescence polarization; lipid membrane fluidity; pentachlorophenol (PCP); *Sphingomonas* sp. UG30.

INTRODUCTION

Pentachlorophenol (PCP) and sodium pentachlorophenolate (NaPCP) have been used extensively in North America to treat railway ties and hydro poles [1,2] and as an insecticide, fungicide and herbicide [3,4]. Because of its widespread use, PCP contamination has been found in sediments, soil, water and sometimes in the atmosphere [3–5]. PCP is lipophilic and can accumulate in plant, animal and human tissues [5–8]. It is also embryotoxic, teratogenic and a suspected carcinogen [5,9] and as a result, has been designated a priority pollutant by the USEPA [9], European Community and Canada [11,12]. The high degree of chlorination makes PCP resistant to biological degradation or physico-chemical modification. However, some microorganisms, mostly isolated from PCP-contaminated soils, can mineralize PCP [4]. *Sphingomonas* sp. UG30 was first isolated by our group at the University of Guelph for its ability to mineralize high concentrations (up to 250 ppm) of NaPCP in minimal salts (MS) medium and up to 300 ppm in MS medium supplemented with glutamate [13].

PCP is known to uncouple oxidative phosphorylation [14,15] and both PCP and NaPCP have been shown to change the membrane phase in model [16,17] and bacterial membranes [18]. UG30 membranes become de-energized on contact with non-lethal concentrations (120 μg/mL or 120 ppm) of NaPCP [19], suggesting membrane function is affected. As the membrane is the first point of contact between the bacterium and its external environment, some environmental conditions (i.e., exposure to chemicals or changes in temperature, ions, pressure or nutrients) experienced by the cell may alter membrane structure and function. Under physiological conditions, membrane fluidity is maintained within certain limits to allow the membrane to function optimally [20]. Changes in bacterial membrane fluidity upon exposure to xenobiotics has been reviewed [21]. Very few studies have investigated

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ABBREVIATIONS: 1,6 diphenyl-1,3,5-hexatriene, DPH; 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl]phenylpropionic acid, DPH-PA; minimal salts, MS; media; minimal salts plus glutamate, MSG; media; pentachlorophenol, PCP; sodium pentachlorophenolate, NaPCP; tetrahydrofuran, THF; 2,2′,5,5′-tetrachlorobiphenyl, TeCB.

1053-0509/03/0900-0385/0

the effects of a xenobiotic compound on the membranes of degrading bacteria. In this paper, we examined if short-term and long-term exposure to PCP and NaPCP affected the cytoplasmic membrane of the PCP-mineralizing bacterium, *Sphingomonas* sp. UG30. This was studied using cellular fatty acid composition analysis and fluorescence polarization of membranes in intact cells.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Minimal salts plus glutamate (MSG) or minimal salts (MS) growth media were used for cultivation of *Sphingomonas* sp. UG30 as described by Leung *et al.* [13]. Cells were grown to mid-exponential phase (21 hr) in a 250 mL Erlenmeyer flask, at 30˚C from a 2% (v/v) inoculum of mid-exponentially growing cells shaken at 200 rpm. *Ralstonia eutropha* H850 was grown as described by Bedard *et al.* [22]. *R. eutropha* H850 was used as a control species in our membrane studies based on our recently published research [23,24]. For membrane polarization studies with H850 cells, fructose (0.1% w/v) was used as the sole carbon source and cells were aseptically harvested during mid-exponential phase as described by Kim *et al.* [23,24].

Chemicals

Acetone and tetrahydrofuran (THF) were obtained from Fisher Scientific (Toronto, ON, Canada). Fructose was purchased from Sigma Chemical Company (St. Louis, MO, USA). PCP and NaPCP were purchased from Aldrich Chemical Company (Milwaukee, WI, USA) and TeCB (2,2′,5,5′-tetrachlorobiphenyl) was obtained from Accustandard Inc. (New Haven, CT, USA). The fluorescent probes DPH (1,6 diphenyl-1,3,5-hexatriene) and DPH-PA (3-[*p*-(6-phenyl)-1,3,5-hexatrienyl]phenylpropionic acid) and the stain DAPI (4′6-diamidino-2-phenylindole dihydrochloride) were obtained from Molecular Probes Inc. (Eugene, OR, USA).

Membrane Fluidity Determinations

Bacterial cultures were grown according to the conditions described above. Aliquots (15 mL) of cells from the various growth treatments were aseptically removed. Bacteria were harvested aseptically by centrifugation at 6,400 × *g* for 10 min at 21°C and washed three times with sterile 15 mM Tris-HCl buffer, pH 7.0. In studies comparing the membrane polarization of H850 and UG30 cells treated with TeCB, PCP or NaPCP, cells were resuspended in sterile potassium phosphate buffer (50 mM, pH 7.0). All samples were adjusted to an optical density of 0.40 at 600 nm and used immediately for fluorescence polarization measurements.

Fluorescence polarization was determined using the membrane probes DPH and DPH-PA as described by Kim *et al.* [23,24] with some modifications. One μL of each probe from a 12 mM stock solution prepared in THF was added to separate 3 mL cell suspensions to a final probe concentration of 4 μM. Initial experiments showed that 10 min of incubation in the dark, with magnetic stirring, was sufficient to allow for DPH and DPH-PA incorporation into the membranes. Fluorescence polarization was measured using a PTI spectrofluorometer (Photon Technology Inc., London, ON, Canada) equipped with a stirrer and a thermostated cuvette holder. The excitation and emission wavelengths were 358 and 428 nm, respectively, for DPH and 364 and 430 nm, respectively, for DPH-PA. Slit widths were 11 and 6 nm for excitation and emission, respectively. Blank samples (with bacteria but no fluorescent probe added) were recorded to account for light scattering. The effect of the probe solvent, THF, on the membranes was found to be negligible. Fluorescence polarization ratio (P) was calculated according to Shinitzky and Barenholz [25]: $P = (I_{vv} - I_{hv}G)/(I_{vv} + I_{hv}G)$, where I_{vv} and I_{hv} are the intensities of emitted light polarized in a parallel and perpendicular direction with respect to the exciting beam of light [26,27]. The grating factor (G) is the correction factor for the photomultiplier tubes [28] and is the ratio of vertically to horizontally polarized light when the exciting beam of light is polarized horizontally [29,30]. The rotational constraint of the probe was determined by depolarization of the exciting beam as the membrane probes rotated [31]. Membrane fluidity decreases when the membrane lipids become more rigid or gel-like causing the polarization ratio to increase due to the constraint exerted upon the membrane probe [26,27]. When membrane lipids are in the liquid-crystalline phase, a greater degree of fluidity prevails and increased rotation of the membrane probe can occur, resulting in a lower polarization ratio.

Fatty Acid Compositional Analysis

Total cellular fatty acids were extracted according to the protocol provided by Microbial ID, Inc. (Newark, DE, USA) [32]. Fatty acids were saponified, methyl esterified and the fatty acid methyl esters analyzed by GC-FID using MIDI microbial identification as described by Kim *et al.* [23,24].

Statistical and Data Analysis

All experiments were conducted in triplicate. Values presented are the averages ± SD. Statistical analysis was performed using two-way ANOVA using SigmaStat 2.0 (Jandel Scientific Software).

RESULTS AND DISCUSSION

Probe Incorporation into Membranes

Initial experiments were undertaken at 21°C to determine the optimal concentration of UG30 cells to use in the fluorescence polarization experiments at a final probe concentration of 4 μM. As the cell concentration increased the DPH polarization ratio increased, but leveled off at OD_{600nm} of 0.6. In theory, the DPH polarization should not change as a function of cell concentration. The DPH probe may have been more susceptible to light scattering by cells and/or endogenous cell fluorophores. The polarization ratio of the DPH-PA probe did not vary in response to changing cell concentration ranging from an OD_{600nm} of 0.05 to 0.8. For subsequent experiments, an OD_{600nm} of 0.4 was used as this allowed a detectable DPH fluorescence polarization value while still being dilute enough to minimize light scattering.

The effect of varying DPH concentrations (1–10 μM) was assessed at a fixed number ($OD_{600nm} = 0.4$) of UG30 cells. No change in fluorescence polarization was observed as the DPH concentration varied. For subsequent experiments, 4 μM of each probe was used. This yielded the maximum fluorescence when used with *Sphingomonas* sp. UG30 cells (data not shown), and was in the same magnitude of probe concentration used by Fabra de Peretti *et al.* [33], 1.5 μM, Moya-Quiles *et al.* [34], 2 μM, Kim *et al.* [23,24], 4 μM. Antunes-Madeira and Madeira [26,35]; Antunes-Madeira *et al.* [36]; Donato *et al.* [37–39] used a probe concentrations ranging from 0.86 to 1.75 μM.

Direct Effects of NaPCP and PCP on *Sphingomonas* sp. UG30 Membranes

The direct effects of varying PCP and NaPCP concentrations on UG30 membranes were examined to determine if the different solubility and partitioning properties of PCP and NaPCP had differential effects on membrane fluidity. NaPCP becomes disassociated upon contact with water, rendering it more water soluble. PCP is more lipophilic and can partition to a greater extent in the bilayer core.

Previous studies in our research program showed that UG30 can mineralize NaPCP up to 250 ppm in minimal salts (MS) medium and up to 300 ppm NaPCP in MS medium supplemented with glutamate [13,40]. Other studies have found membrane changes occurring at lower or within this range of PCP or NaPCP concentrations [14,18,41,42]. In particular, concentrations of 120 μg/mL (120 ppm) NaPCP caused decreased ATP levels and transmembrane pH gradient in immobilized UG30 cells, as well as an increase in the cardiolipin composition of the membranes [19].

UG30 cells were harvested, washed and 50–250 ppm of PCP or 50–1000 ppm NaPCP were added along with either DPH or DPH-PA. No changes in the fluorescence polarization ratios were observed when UG30 membranes were directly exposed to 50–250 ppm NaPCP or PCP as monitored by DPH or DPH-PA fluorescence at 21°C (data not shown). Concentrations of NaPCP greater than 250 ppm were tested in our study as Suwalsky *et al.* [17] observed alterations to artificial dismyristoylphosphatidylethanolamine (DMPE) and dimyristoylphosphatidyl-choline (DMPC) membrane structure after exposure to 0.02 M (5767 ppm) NaPCP. In our study, while higher polarization values were obtained when UG30 cell membranes were exposed to 1000 ppm NaPCP, we believe this was due to the effect of turbidity and increased light scattering. NaPCP concentrations above 1000 ppm made the suspension in the cuvette turbid, rendering it difficult to obtain accurate fluorescence polarization measurements. As PCP was dissolved in acetone, the effect of this solvent on fluorescence polarization was also examined. No differences were found between the UG30 cultures exposed to only acetone or those exposed to both acetone and PCP added (data not shown). As there were no significant differences between the effects of PCP and NaPCP, the soluble sodium salt was used in the remainder of the studies.

Membrane Fluidity of *Sphingomonas* sp. UG30 Cultures Degrading NaPCP

During direct exposure of UG30 membranes to NaPCP, no changes in membrane fluidity were evident. The question arises as the whether membranes were susceptible to fluidity changes when exposed to NaPCP for a longer period of time and during degradation of NaPCP. The fluorescence polarization ratio of membranes of UG30 cells growing in MS or MSG media supplemented with 25 and 50 ppm NaPCP was determined. We previously observed that concentrations of 25 and 50 ppm NaPCP could be degraded by UG30 within 6 hr (data not

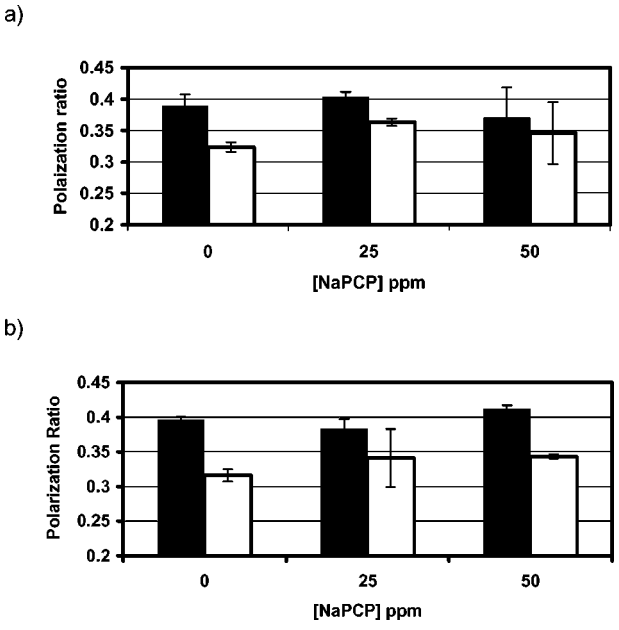

Fig. 1. Fluorescence polarization ratio of membranes of *Sphingomonas* sp. UG30 in minimal salts (MS) media during degradation of NaPCP after 3 hr (■) and 6 hr (□) as measured by (a) DPH and (b) DPH-PA. Values are mean ± SD ($n = 3$).

shown). Neither the DPH nor DPH-PA probe detected any membrane fluidity changes in the NaPCP-degrading cultures in MS media after 3 or 6 hr, relative to the control (Fig. 1a and b). Similarly, no changes were observed in the fluorescence polarization ratio of the cultures degrading NaPCP in the MSG-NaPCP media using the DPH or DPH-PA probes over the 3 and 6 hr time periods (data not shown). It is currently not known why the 6 hr fluorescent polarization readings were lower than those measured at 3 hr.

During this study, UG30 cultures grown in MS-NaPCP and MSG-NaPCP media were harvested for total cellular fatty acid analysis. No significant changes in the ratio of saturated to unsaturated fatty acids or straight chain fatty acids were observed in either media with 25 or 50 ppm NaPCP present. The only differences observed were those related to the composition of branched fatty acids. Less branched fatty acids were found in MS-grown cultures exposed to 50 ppm NaPCP for 3 hr (8.4% ± 0.8) as compared to cells exposed to 25 ppm (12.4% ± 0.1) or to the control (11.8% ± 1.0) (Table I). After 6 hr, control cells grown in MSG had a higher percentage of branched fatty acids (10.0% ± 0.7) than cells exposed to 25 ppm NaPCP (7. 7% ± 0.4) (Table II). Decreased levels of branched fatty acids were observed in cultures exposed to NaPCP. This decrease in branched fatty acid could serve to decrease fluidity. We did not observe any changes in saturated to unsaturated fatty acid ratios or fluorescence polarization values between different treatments.

Overall, we did not find any alterations in the structure of *Sphingomonas* sp. UG30 membranes after short-term (immediate) or longer term exposure to PCP or NaPCP using fluorescence polarization measurements and fatty acid composition analysis. Even when a longer exposure time (24 hr) was used, no change in membrane fluidity was observed (data not shown). A lipid soluble compound such as PCP would have been hypothesized to have an effect on the membrane of this bacterium. Other studies have shown that treatments with PCP affect membrane fluidity. A fluid to gel-phase shift in the membrane of *Pseudomonas fluorescens* was observed by X-ray diffraction, after treatment with 25 μg/mL

Table I. Percentages of Fatty Acids in the Membranes of *Sphingomonas* sp. UG30 in Minimal Salts (MS) Media During Degradation of NaPCP after 3 and 6 hr at 30°C

	% of total fatty acids					
	Control		25 ppm NaPCP		50 ppm NaPCP	
Fatty acids	3 hr	6 hr	3 hr	6 hr	3 hr	6 hr
Total straight	10.2 ± 0.71	10.0 ± 1.5	9.85 ± 0.2	10.1 ± 1.3	9.91 ± 0.3	8.9 ± 0.69
Total *cis*-unsaturated	68.1 ± 0.64	69.3 ± 1.4	69.9 ± 0.7	69.7 ± 1.9	69.5 ± 1.2	67.8 ± 0.37
Total branched	11.8 ± 0.96	11.5 ± 0.7	12.4 ± 1.0	11.9 ± 0.5	8.44 ± 0.8	15.4 ± 0.85
Total cyclopropane	0.18 ± 0.16	0.51 ± 0.2	0.20 ± 0.2	0.42 ± 0.1	0.19 ± 0.2	0
Total *iso*-branched	0	0	0.05 ± 0.1	0.04 ± 0.1	0.16 ± 0.2	0.6 ± 0.6
[a]Total saturated	10.3 ± 0.9	10.2 ± 1.5	10.1 ± 0.3	10.1 ± 1.3	9.91 ± 0.3	8.9 ± 0.7
[b]Total unsaturated	89.7 ± 0.9	89.8 ± 1.5	90.3 ± 1.0	89.9 ± 1.3	90.1 ± 0.3	91.1 ± 0.7
Saturated/unsaturated	11.5 ± 1.1	11.3 ± 1.8	11.1 ± 0.3	11.3 ± 1.6	11.0 ± 0.3	9.7 ± 0.8

Note. Values are mean ± SD ($n = 3$).
[a]Total saturated fatty acids including straight- and cyclopropane chains.
[b]Total unsaturated fatty acid including hydroxylated chains.

Table II. Percentages of Fatty Acids in the Membranes of *Sphingomonas* sp. UG30 in Minimal Salts and Glutamate (MSG) Media During Degradation of NaPCP after 3 and 6 hr at 30°C

	% of total fatty acids					
	Control		25 ppm NaPCP		50 ppm NaPCP	
Fatty acids	3 hr	6 hr	3 hr	6 hr	3 hr	6 hr
Total straight	12.8 ± 0.9	15.0 ± 4.5	12.2 ± 0.3	12.1 ± 0.4	10.9 ± 0.1	10.1 ± 1.1
Total *cis*-unsaturated	65.6 ± 0.9	65.2 ± 4.1	67.7 ± 0.3	67.9 ± 0.6	68.7 ± 0.6	69.5 ± 1.0
Total branched	9.4 ± 1.2	10.0 ± 0.7	10.3 ± 0.9	7.7 ± 0.4	9.90 ± 50.5	9.99 ± 0.5
Total cyclopropane	0	0.04 ± 0.1	0	0	0	0
Total *iso*-branched	0	0	0	0	0.49 ± 0.1	0.06 ± 0.10
[a]Total saturated	12.8 ± 0.9	15.1 ± 4.45	12.2 ± 0.3	12.1 ± 0.4	10.9 ± 0.1	10.1 ± 1.1
[b]Total unsaturated	87.0 ± 1.2	84.9 ± 4.45	87.8 ± 0.3	87.9 ± 0.4	89.1 ± 0.2	89.9 ± 1.2
Saturated/unsaturated	14.7 ± 1.2	18.0 ± 6.4	13.9 ± 0.4	13.8 ± 0.5	12.2 ± 0.2	11.2 ± 1.4

Note. Values are mean ± SD ($n = 3$).
[a]Total saturated fatty acids including straight- and cyclopropane chains.
[b]Total unsaturated fatty acid including hydroxylated chains.

(25 ppm) PCP for 12 hr [18]. Membranes of Chinese hamster fibroblasts showed decreased fluorescence polarization ratios of 35 and 50% when probed with DPH and *trans*-parinaric acid, respectively, after treatment with 282 μM (81.3 ppm) of PCP for 24 hr [42]. In artificial dismyristoylphosphatidylethanolamine (DMPE) and dimyristoylphosphatidyl-choline (DMPC) bilayers, exposure to 0.02 M (5767 ppm) NaPCP for 4 hr was found to perturb both bilayer structures as indicated by X-ray diffraction and DPH fluorescence anisotropy [17]. Fluidization of the DMPC and DMPE bilayers was postulated to occur with PCP ions intercalating between the phospholipid polar head groups [17].

2.4.7 Direct Effects of PCP, NaPCP, and 2,2′,5,5′-Tetrachlorobiphenyl (TeCB) on the Membranes of *Ralstonia eutropha* H850 and *Sphingomonas* sp. UG30

During our research into the effects of NaPCP on UG30 membranes, no adverse effects were observed. One possible reason for the lack of effect may be that the changes in the UG30 membranes were too subtle to be detected by the methods employed. The lack of effect of PCP or NaPCP on UG30 membranes raised questions as to the suitability and accuracy of our methodology. If the methods used were suitable, then our results may suggest that the UG30 membranes are resistant to fluidization by PCP and NaPCP and/or PCP may be a poor fluidizer of biological membranes.

We have previously shown that membranes of *Ralstonia eutropha* H850 can be fluidized by direct exposure to low concentrations of TeCB (2,2′,5,5′-tetrachlorobiphenyl) and this fluidization could be measured by fluorescence polarization [23]. To confirm the suitability of the methods, we performed two experiments to determine: (a) if the UG30 membranes could be fluidized by TeCB; and (b) if NaPCP could perturb the membranes of *Ralstonia eutropha* H850. As expected and in agreement with Kim *et al.* [23], TeCB at 1.46 and 2.92 ppm fluidized H850 membranes, as measured by a significant decrease (ANOVA, $p = 0.05$) in the polarization ratio (Fig. 2a). TeCB also fluidized the UG30

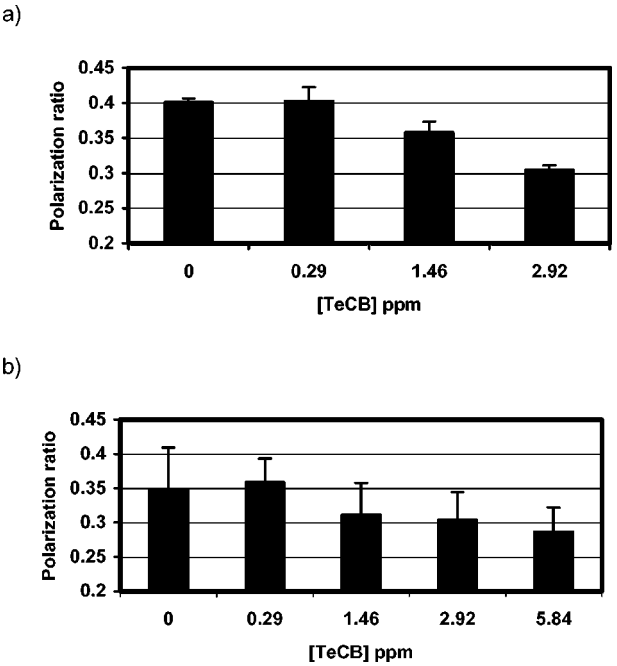

Fig. 2. Direct effects of 2,2′,5,5′-tetrachlorobiphenyl (TeCB) on (a) *Ralstonia eutropha* H850 and (b) *Sphingomonas* sp. UG30 membranes as measured by the DPH fluorescence polarization ratio. Values are mean ± SD ($n = 3$).

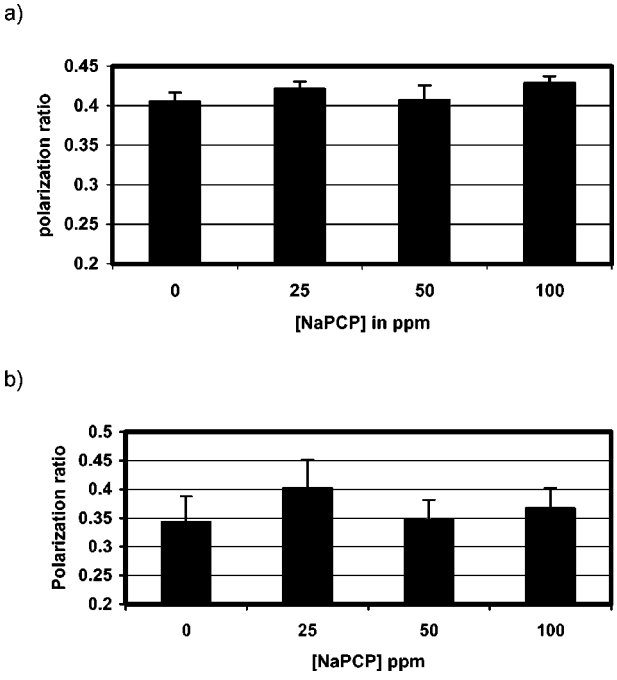

Fig. 3. Direct effects of NaPCP on (a) *Ralstonia eutropha* H850 and (b) *Sphingomonas* sp. UG30 membranes as measured by DPH fluorescence polarization. Values are mean ± SD ($n = 3$).

membranes although the values were not statistically significant (ANOVA, $p = 0.05$) even at a higher TeCB concentration (5.84 ppm). The results showed that the UG30 membranes were fluidized by TeCB, although they were slightly more resistant than H850 membranes. The results also indicated the suitability of our fluorescence polarization method and suggested that the lack of effect of NaPCP may be due to the poor ability of this compound to fluidize biological membranes. As confirmation, NaPCP at 100 ppm did not significantly alter the polarization ratio or either H850 or UG30 membranes (Fig. 3a and b). Interestingly, the membranes of UG30 had a saturated to unsaturated fatty acid ratio of 11.5–14.7% (Tables I and II) as compared to the 50% ratio in H850 membranes [23]. The lower amount of saturated fatty acids in the UG30 membranes may have resulted in the membrane being less easily perturbed by NaPCP. While our research shows that the membranes of UG30 are more resistant to fluidization than *Ralstonia eutropha* H850 membranes, we cannot rule out that NaPCP does not disturb membrane structure in either of the microorganisms or other microorganisms not capable of degrading NaPCP. It may be that the effects of NaPCP were too subtle to be detected by the methods employed and/or *Sphingomonas* sp. UG30 membranes are not affected by PCP and NaPCP, which it can easily mineralize. However, to our knowledge the fluorescent polarization values reported in this study and in the review by Denich *et al.* [21] are the first available for a *Sphingomonas* sp.

ACKNOWLEDGMENTS

We thank the Canadian Chlorine Coordination Committee (C4) and the Canadian Chemical Producers Association (CCPA) for funding our studies on membrane-pollutant interactions. We gratefully acknowledge the infrastructure support from the Canadian Foundation for Innovation (CFI) and the Ontario Challenge Fund. Research by HL and JTT was also supported by Discovery grants from the Natural Sciences and Engineering Research Council (NSERC) of Canada.

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